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Sterols Accelerate Degradation of Hamster 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Encoded by a Constitutively Expressed cDNA

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A recombinant plasmid containing a full-length cDNA for hamster 3-hydroxy-3-methylglutaryl coenzyme A reductase was introduced by calcium phosphate-mediated transfection into UT-2 cells, a mutant line of Chinese hamster ovary cells that lack 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and thus require low density lipoprotein-cholesterol and mevalonate for growth. We selected a line of permanently transfected cells, designated TR-36 cells, that expressed high levels of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and thus grew in the absence of low density lipoprotein and mevalonate. Constitutive synthesis of reductase mRNA in TR-36 cells was driven by the simian virus 40 early promoter, and therefore the mRNA was not suppressed by sterols, such as 25-hydroxycholesterol or cholesterol derived from low density lipoprotein, which normally suppresses transcription of reductase mRNA when the reductase gene is driven by its own promoter. Although TR-36 cells continued to synthesize large amounts of reductase mRNA and protein in the presence of sterols, reductase activity declined by 50 to 60%. This decline was caused by a twofold increase in the rate of degradation of preformed enzyme molecules. The current data demonstrate that sterols accelerate the degradation of reductase protein independently of any inhibitory effect on the synthesis of the protein.

The endoplasmic reticulum enzyme 3-hydroxy-3-methylglutaryl (HMG) coenzyme A (CoA) reductase catalyzes the rate-determining step in the synthesis of cholesterol and other polyisoprenoid compounds in animal cells. The activity of the enzyme is subject to feedback suppression by several agents, including cholesterol derived from the receptor-mediated endocytosis of plasma low density lipoprotein (LDL); oxygenated sterols (such as 25-hydroxycholesterol) added to cells in solvents; and nonsterol substances synthesized from mevalonate, the product of the HMG CoA reductase reaction (for a review, see reference 3). Recently, a full-length cDNA for hamster HMG CoA reductase was isolated, and the amino acid sequence of the protein was deduced from the nucleotide sequence of the cDNA (5). Using this cDNA as a probe, we demonstrated that the feedback regulation of HMG CoA reductase by LDL-cholesterol in cultured hamster cells is mediated in part by a reduction in the level of mRNA for reductase, owing to a decrease in the rate of transcription of the gene (19, 26). Similar results have been reported for cultured cells by Hardeman et al. (15), and a similar parallelism between the level of reductase activity, reductase protein, and reductase mRNA was also demonstrated in rat livers by Liscum et al. (17) and Clarke et al. (8).

Although the major point of regulation of HMG CoA reductase appears to be at the level of mRNA transcription, recent data have begun to suggest that in addition to decreasing the synthesis of new HMG CoA reductase molecules, sterols and other mevalonate-derived substances also increase the rate of proteolytic degradation of preexisting enzyme molecules (9, 10, 29). This effect of sterols on reductase degradation is apparent in UT-1 cells, a line of Chinese hamster ovary (CHO) cells that was selected for resistance to compactin, a competitive inhibitor of HMG CoA reductase (6, 10). UT-1 cells have a 15-fold amplification of the gene for HMG CoA reductase, and they also transcribe each gene more efficiently, so that they have a greater than 200-fold increase in the amount of reductase mRNA and protein (19). The increased amount of enzyme is housed in a system of membrane tubules called crystalloid endoplasmic reticulum (6, 25). When sterols are added to UT-1 cells, the entire crystalloid endoplasmic reticulum, as well as HMG CoA reductase protein, undergoes rapid degradation (25).

The mechanism of the accelerated degradation of reductase has been difficult to study because it is always accompanied by a profound decrease in synthesis of new reductase molecules. An understanding of the degradative mechanism would be facilitated by the availability of cells in which the effects of sterols on enzyme degradation could be examined independently of their effects on mRNA transcription and synthesis of the enzyme. In this study, we created such a cell line by taking advantage of UT-2 cells, a line of mutant CHO cells that lack HMG CoA reductase activity and therefore require mevalonate plus LDL-derived cholesterol for growth (22). We used the UT-2 cells as recipients for transfection by pRed-227, a plasmid that contains a cDNA for the coding region of the reductase mRNA (5), and have selected cells that no longer require exogenously added mevalonate and LDL for growth. In pRed-227 the cDNA insert is located downstream of the simian virus 40 (SV40) early promoter (5). cDNA inserts in this vector are known to be transcribed from the SV40 promoter when the plasmid integrates into the genome of animal cells (23, 24). In the current experiments, we found that sterols do not repress the synthesis of

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reductase mRNA or protein in the transfected cells. Yet sterols still accelerate the degradation of preformed enzyme molecules. These data indicate that the sterol-mediated stimulation of degradation and the sterol-mediated inhibition of synthesis of reductase protein are separate events.

MATERIALS AND METHODS

Materials. G-418 sulfate (Geneticin) was purchased from GIBCO Laboratories. pSV3-Neo, a plasmid containing a bacterial gene that confers resistance to G-418 (27), was obtained from Bethesda Research Laboratories. Compactin was a gift from Akira Endo (Tokyo Noko University, Tokyo, Japan) and was used as the sodium salt (1). [α-^{32}P]dNTP (3000 Ci/mmol), [^{35}S]methionine (986 to 1084 Ci/mmol), and DL-3-hydroxy-3-methyl-[3-^{14}C]glutaryl CoA (55 mCi/mmol) were obtained from New England Nuclear Corp. Restriction endonucleases were obtained from New England Biolabs, the Klenow fragment of *Escherichia coli* DNA polymerase I was obtained from Boehringer Mannheim Biochemicals, and S1 nuclease was obtained from Miles Laboratories, Inc. pRed-227, a recombinant plasmid containing a 4.5-kilobase (kb) cDNA for hamster HMG CoA reductase, was prepared as described previously (5). Newborn calf lipoprotein-deficient serum ($d > 1.215$ g/ml) and human LDL ($d = 1.019$ to 1.063 g/ml) were prepared by ultracentrifugation (12). A rabbit polyclonal antibody directed against the 53-kilodalton COOH-terminal domain of hamster HMG CoA reductase was prepared as described previously (6). Other materials were obtained from previously described sources (19, 22, 26).

Cell culture. All cells were grown in monolayer cultures at 37°C in an atmosphere of 5 to 7% CO_2. Stock cultures of CHO cells were grown in medium A (Ham F-12 medium containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.4], 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2mM glutamine) supplemented with 10% (vol/vol) fetal calf serum. Stock cultures of UT-1 cells (6) were grown in medium A supplemented with 8% lipoprotein-deficient serum–2% fetal calf serum–40 μM compactin. Stock cultures of UT-2 cells (22) were grown in medium A supplemented with 10% fetal calf serum–0.2 mM mevalonate. Stock cultures of TR-36 cells (see below) were grown in medium A supplemented with 8% lipoprotein-deficient serum–2% fetal calf serum–1 μM compactin.

Cells were set up for assays of HMG CoA reductase activity, immunoprecipitable ^{35}S-labeled reductase, and cholesterol esterification by a standard format. Cells from stock flasks were dissociated with trypsin-EDTA. On day 0, 3×10^4 CHO cells, 7×10^4 TR-36 cells, or 7×10^4 UT-1 cells were seeded into each Petri dish (60 by 15 mm) in 3 ml of medium A supplemented with 8% lipoprotein-deficient serum–2% fetal calf serum in the absence (CHO and TR-36 cells) or presence of 40 μM compactin (UT-1 cells). On days 2 and 3, the cells were refed with medium A supplemented with 10% lipoprotein-deficient serum. UT-1 cells were cultured continuously in the presence of 40 μM compactin. Additions of sterols or LDL were made on day 4 or 5.

For preparations of DNA and RNA, cells were cultured as described above except that the cells were grown in roller bottles or 100- by 15-mm dishes. The number of cells seeded and the volume of medium added were scaled up appropriately (4×10^6 to 8×10^6 cells and 75 ml of medium per roller bottle and 0.5×10^5 to 1.5×10^5 cells and 8 ml of medium per 100-mm dish).

Isolation of TR-36 cells. Plasmid pRed-227 was introduced into the UT-2 cell line, a mevalonate auxotroph (22), by cotransfection with pSV3-Neo by the calcium phosphate precipitation method (30). Briefly, on day 0, 10^6 UT-2 cells were seeded into 100- by 15-mm Petri dishes containing 12 ml of medium B (medium A supplemented with 10% fetal calf serum and 0.2 mM mevalonate). On day 1, a calcium phosphate coprecipitate of pRed-227 (8 μg) and pSV3-Neo (4 μg) was added to the medium of each dish. On day 2, the cells were washed twice with Dulbecco phosphate-buffered saline, incubated 4 min at 37°C with 5.5 ml of medium B supplemented with 10% (vol/vol) glycerol, washed three times with phosphate-buffered saline, and then cultured with medium B. On day 3, the cells were trypsinized and replated at a density of 2×10^5 cells per 100-mm dish in medium B. On day 4, the cells were washed with phosphate-buffered saline and incubated with selective medium, which was composed of medium A supplemented with 10% lipoprotein-deficient serum and 200 μg of G-418 per ml and lacking mevalonate. Fresh selective medium was added every 4 days. Visible clones were picked after 23 days and expanded by culturing in medium A supplemented with 10% fetal calf serum and 200 μg of G-418 per ml. One of these clones, designated TR-36 cells, was adapted over a 6-week period to growth in medium A supplemented with 8% lipoprotein-deficient serum–2% fetal calf serum–100 μg of G-418 per ml–1 μM compactin. For 12 months, TR-36 cells were maintained in medium A supplemented with 8% lipoprotein-deficient serum–2% fetal calf serum–1 μM compactin.

Immunoprecipitation of ^{35}S-labeled reductase. Cell monolayers were incubated with [^{35}S]methionine as described in the legends to the figures. The cells were washed at 4°C with 50 mM Tris-hydrochloride (pH 7.4)–150 mM NaCl, scraped into 1 ml of the same buffer, and centrifuged at $12,000 \times g$ for 30 s at 4°C. The cell pellet was suspended by blending with a Vortex mixer in 0.1 ml of 10 mM potassium phosphate (pH 7.5)–5 mM EDTA–5 mM EGTA–0.1 M NaCl–1% (vol/vol) Triton X-100–0.1% (wt/vol) sodium dodecyl sulfate–0.01% (wt/vol) NaN_3–2 mM phenylmethylsulfonyl fluoride–0.5% (wt/vol) sodium deoxycholate–50 mM dithiothreitol–100 μM leupeptin. After 20 min at 4°C, the extract was centrifuged at $12,000 \times g$ for 5 min at 4°C. Separate portions of the supernatant were removed for protein determination, precipitation with trichloroacetic acid, and immunoprecipitation as previously described (10). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the ^{35}S-labeled immunoprecipitates and autoradiography were performed as described previously (10).

Blot hybridization of DNA. Genomic DNA was prepared as previously described (19, 20). Genomic DNA was digested with restriction endonucleases, extracted with phenol-chloroform (1:1) and then ether, precipitated with ethanol, and subjected to electrophoresis in a 0.8% agarose gel containing 30 mM Tris-hydrochloride (pH 8.15), 20 mM sodium acetate, 2 mM NaCl, and 2.5 mM EDTA (20). ^{32}P-labeled DNA hybridization probes were generated by nick-translation (20) or by mixed oligonucleotide primer extension with [α-^{32}P]dCTP and the Klenow fragment of *E. coli* DNA polymerase I (11). Prehybridizations and hybridizations were performed at 43°C in 50% (vol/vol) formamide, 4× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate at pH 7.2), 0.1 μg of denatured calf thymus DNA per ml and 5× Denhardt solution (1× Denhardt solution is 0.01% [wt/vol] bovine serum albumin, 0.01% [wt/vol] Ficoll, 0.01% [wt/vol] polyvinylpyrrolidone) (20). Blots were washed free of nonspecifically bound radioactivity in 0.1× SSC and 0.2 to 1% (wt/vol) sodium dodecyl sulfate for 1 h at 52°C.

Measurement of reductase mRNA by DNA-excess solution hybridization. The content of reductase mRNA in cultured cells was measured by DNA-excess solution hybridization with minor modification of the method described by Williams et al. (D. L. Williams, T. C. Newman, G. S. Shelness, and D. A. Gordon, Methods Enzymol, in press). Total cellular RNA was isolated from CHO and TR-36 cells by guanidine hydrochloride extraction (28). An M13 DNA template was prepared by subcloning a 182-base-pair (bp) *Pst*I-*Pst*I fragment (nucleotides 1527 to 1709) from the coding region of pRed-227 (5) into the *Pst*I site of the M13mp8 vector (21). A single-stranded cDNA probe for reductase was synthesized by using M13 DNA as a template; a universal sequencing primer of 17 nucleotides in length; 0.25 mM dTTP, dATP, and dGTP; 7.4 μM [α-^{32}P]dCTP; and the Klenow fragment of *E. coli* DNA polymerase I. After incubation for 1 h at 37°C, unlabeled dNTPs were added at a final concentration of 0.25 mM for 10 min at 37°C. The single-stranded fragment was isolated by *Eco*RI digestion followed by 7 M urea–5% polyacrylamide gel electrophoresis and hydroxylapatite chromatography (Williams et al., in press). The single-stranded [^{32}P]cDNA probe (35 fmol, ~1.7 × 10^4 cpm/fmol) was hybridized with various amounts of total cellular RNA in 0.1 ml of 20 mM Tris-hydrochloride (pH 7.8)–20 mM EDTA–0.3 M NaCl–100 μg of denatured salmon sperm DNA per ml at 68°C for 60 h. The sample was digested with S1 nuclease, and hybrids were collected by precipitation with 7.5% trichloroacetic acid. The specific radioactivity of the [^{32}P]cDNA probe was calculated by hybridization with a known amount of M13 template, followed by S1 nuclease digestion and acid precipitation.

Other assays. Protein was measured by a modified procedure described by Lowry et al. (18). Enzymatic activity of HMG CoA reductase was measured in detergent-solubilized cell extracts (0.1 to 40 μg of protein per assay) as described previously (12). One unit of reductase activity represented the formation of 1 nmol of [^{14}C]mevalonate per min at 37°C. The incorporation of [^{14}C]oleate into cholesteryl [^{14}C]oleate by cell monolayers was determined as described previously (12).

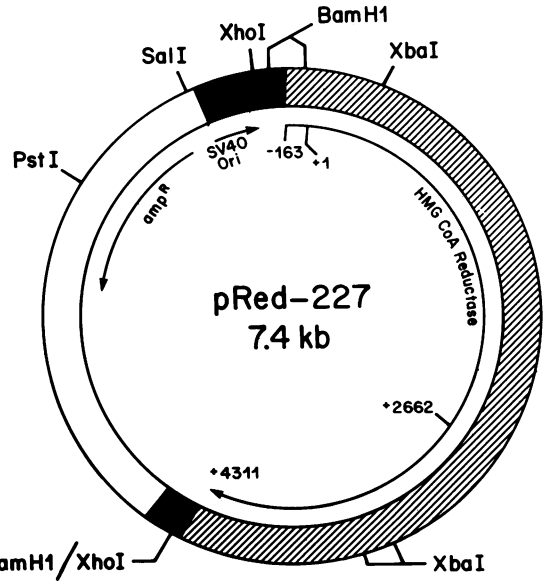

FIG. 1. Structure of pRed-227. pRed-227 was isolated from a cDNA library derived from the polyadenylated RNA of UT-1 cells with a cloning vector that allows for expression in mammalian cells (5). This plasmid contains the entire coding region of HMG CoA reductase (cross-hatched area). The initiator methionine codon (position 1) and terminator codon (position 2662) are indicated. Solid areas denote regions in the cloning vector that contain SV40 sequences, including the origin of replication, 16S and 19S donor and acceptor splicing sites, and polyadenylation signals (23, 24). The open area denotes pBR322 sequences. The sites for relevant restriction endonucleases are indicated.

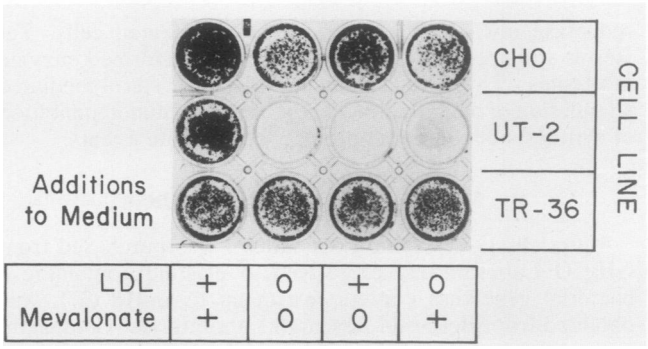

FIG. 2. Stained CHO, UT-2, and TR-36 cells after growth under various conditions. On day 0 of cell growth, the indicated cells were seeded at 3 × 10^3 to 5 × 10^3 cells per well in 24-well Linbro plates in 1 ml of medium A supplemented with 10% fetal calf serum–0.25 mM mevalonate. Six hours after plating the cells, the medium was removed and replaced with 1 ml of medium A containing 10% lipoprotein-deficient serum and the indicated addition of 50 μg LDL per ml or 0.25 mM mevalonate. The cells were refed every second day. On day 7, the cells were washed, fixed with 95% ethanol, and stained with 1% (wt/vol) crystal violet.

RESULTS

The structure of pRed-227 is shown in Fig. 1. The plasmid contains a 4.5-kb cDNA insert that is complementary to 163 bp of the 5′-untranslated region of the reductase mRNA, the entire coding region, and almost 1,700 bp of 3′ untranslated region (5). On the 5′ side of the reductase cDNA, this plasmid contains the SV40 origin of replication, which encompasses the early region promoter. At the 3′ side of the coding region there is a polyadenylation site from SV40. Transcripts originating from this vector initiate in the SV40 early region, which contains an intervening sequence that is spliced out of the mature mRNA (23, 24).

pRed-227 was introduced by calcium phosphate-mediated transfection into UT-2 cells, which lack functional HMG CoA reductase activity (22). Simultaneously, we transfected a plasmid containing the gene for G-418 (neomycin) resistance, also under the direction of the SV40 promoter (27). We selected colonies simultaneously for resistance to G-418 and for growth in the absence of exogenous mevalonate. The parental UT-2 cells, which lack reductase activity, cannot grow in the absence of mevalonate and LDL (Fig. 2). The cell line that we eventually isolated, designated TR-36, grows in the absence of LDL and mevalonate, indicating that it expresses HMG CoA reductase activity (Fig. 2). The cells also grew in the presence of G-418, indicating that they also express the gene for neomycin resistance.

To confirm the integration of pRed-227 and pSV3-Neo into the genome of TR-36 cells, genomic DNA was digested with restriction enzymes, subjected to agarose gel electrophoresis, transferred to nitrocellulose, and probed either with a ^{32}P-labeled DNA fragment that hybridizes to only the SV40 origin (SV40-ori probe) or with a ^{32}P-labeled DNA fragment that hybridizes to the 5′ end of the reductase cDNA as well as to part of the SV40 origin (reductase probe) (Fig. 3). Both of these probes would be expected to hybridize to genomic

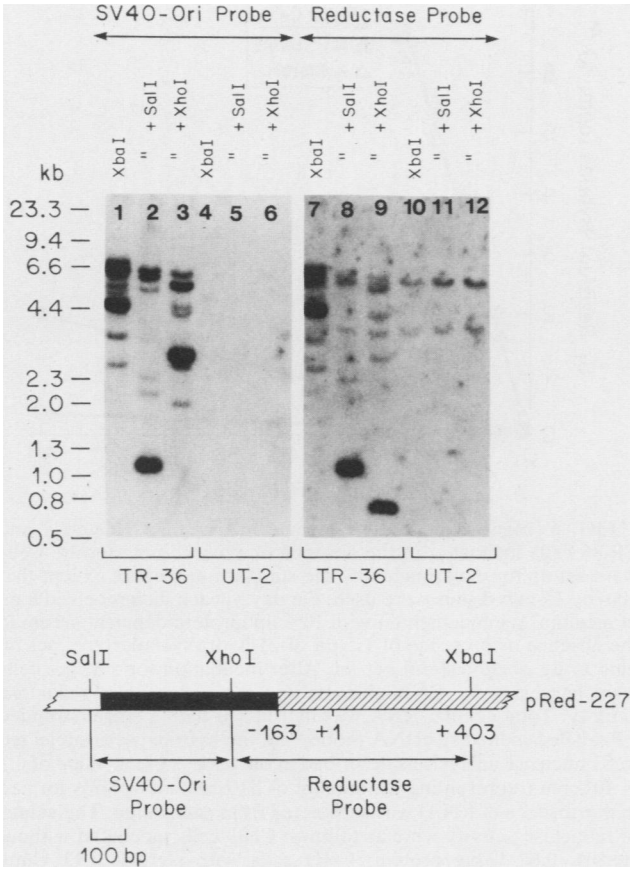

FIG. 3. Blot hybridization of DNA from TR-36 and UT-2 cells. Genomic DNA from TR-36 and UT-2 cells was digested to completion with the indicated restriction endonucleases; and digests (10 μg) were subjected to electrophoresis, transferred to a nitrocellulose filter, and incubated with a ^{32}P-labeled *SalI-XhoI* fragment (426 bp) from pRed-227 (10^6 cpm/ml, 10^8 cpm/μg). The filter was washed and exposed to XAR5 film for 26 h at −70°C with an enhancing screen (lanes 1 through 6). The filter was subsequently washed for 2 h at 70°C in 0.05% (wt/vol) sodium pyrophosphate–10 mM Tris-hydrochloride (pH 8)–0.2 mM EDTA–0.1× Denhardt solution to remove hybridized DNA. After autoradiographic exposure of the stripped filter to verify the removal of radioactivity, the filter was incubated with a ^{32}P-labeled *XhoI-XbaI* fragment (680 bp) from pRed-227 (10^6 cpm/ml, 1.5×10^8 cpm/μg) (lanes 7 through 12) and exposed to film for 24 h. Symbols for the probe are as defined in the legend to Fig. 1.

sequences derived from pSV3-Neo and pRed-227 because both plasmids contain the SV40 origin. When the TR-36 cell DNA was digested with *XbaI* and hybridized with either probe, many fragments were visualized (lanes 1 and 7), indicating that pRed-227 and pSV3-Neo had integrated at multiple sites. As expected, the SV40 ori probe did not hybridize with DNA from UT-2 cells that had not been transfected (lane 4). On the other hand, the reductase probe hybridized to DNA fragments from the nontransfected UT-2 cells (lanes 10 to 12) confirming the earlier observation that the mutant UT-2 cells contain the endogenous reductase gene (22). When the *XbaI*-digested DNA was subjected to double digestion with either *XhoI* or *SalI*, the DNA fragments were reduced in size as expected from the restriction map of pRed-227 (Fig. 1). Of particular importance was the appearance of an intense 1.1-kb fragment in the *XbaI-SalI* digests that hybridized with both the SV40 ori probe (lane 2) and the reductase probe (lane 8). When the DNA was cut with *XbaI* and *XhoI*, this prominent fragment was reduced to 650 bp, and it hybridized only with the reductase probe (lane 9), not the SV40 ori probe (lane 3). This pattern of restriction fragment hybridization confirms that many copies of pRed-227 had integrated in such a way that the SV40 origin and the reductase cDNA remained contiguous.

The measured HMG CoA reductase activity in TR-36 cells ranged between 0.6 and 1.8 U of protein per mg (see legends to Fig. 4 and 6; unpublished data). Under these conditions, the parental UT-2 cells have no detectable reductase activity (22). To determine whether reductase activity in TR-36 cells was susceptible to feedback regulation, we incubated the cells with a mixture of 25-hydroxycholesterol plus cholesterol and compared the response of the TR-36 cells with that of UT-1 cells (Fig. 4A) or CHO cells (Fig. 4B). In studying reductase regulation, we used this mixture of sterols because 25-hydroxycholesterol is more potent than cholesterol in suppressing reductase, but 25-hydroxycholesterol cannot replace cholesterol in maintaining the growth of the cells (2, 13). In both UT-1 and CHO cells, the addition of sterols reduced HMG CoA reductase activity to less than 5% of control values within 18 h. Reductase activity in TR-36 cells was also reduced, but only to about 30 to 40% of control values. A similar partial suppression of reductase was seen when the TR-36 cells were incubated with LDL (data not shown).

To determine whether the sterols were inhibiting the synthesis of reductase protein, we incubated TR-36 cells with sterols and measured with a brief pulse the incorporation of [^{35}S]methionine into immunoprecipitable protein, which was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. As a control we studied UT-1 cells in the same experiment (Fig. 5). As previously described (7, 16, 16a), in UT-1 cells

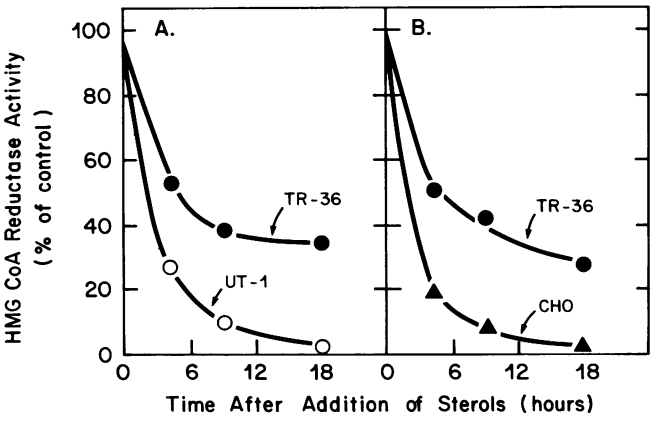

FIG. 4. Suppression of HMG CoA reductase activity in TR-36 (●), UT-1 (○), and CHO (▲) cells after incubation with 25-hydroxycholesterol plus cholesterol for various times. On day 0, cells were set up for experiments by the standard procedure. UT-1 cells were maintained in the continuous presence of 40 μM compactin. Additions of sterols ([A], 1.5 μg of 25-hydroxycholesterol per ml plus 15 μg of cholesterol per ml; [B], 0.5 μg of 25-hydroxycholesterol per ml plus 12 μg of cholesterol per ml) were made in a staggered fashion so that all cells could be harvested at the same time on day 5 after incubation with sterols for the indicated times. The 100% of control values for reductase activity (units per milligram of protein) were as follows: [A], 14 (○) and 1.4 (●); [B], 0.37 (▲) and 1.8 (●). Each value represents the mean of triplicate incubations.

immunoprecipitable reductase showed three predominant bands on sodium dodecyl sulfate-gel electrophoresis (lane B). The major band at 97,000 daltons represents the intact enzyme. A band at about 62,000 daltons represents a proteolytic degradation product. The band at 200,000 daltons and the radioactivity at the top of the gel are believed to be aggregates of the enzyme because they are present in even greater amounts when the immunoprecipitates were boiled or when we did not incubate them for a prolonged period in sodium dodecyl sulfate and urea (7). When UT-1 cells were grown without sterols, there was abundant incorporation of [^{35}S]methionine into all of these immunoprecipitable bands (lane B). When the UT-1 cells had been incubated with sterols for 11 h, the incorporation of radioactivity into all of the bands was markedly diminished (lane D). The TR-36 cells synthesized a spectrum of immunoprecipitable reductase molecules that were similar to those of the UT-1 cells (lane F). Densitometric scanning showed that all of the bands were present in the same proportions as in UT-1 cells with the exception of a minor band at ~250 kilodaltons (13% of radioactivity) that was not present in UT-1 cells. When the TR-36 cells were incubated with sterols, none of the reductase bands decreased by more than 15% (lane H).

Measurements of reductase mRNA levels were consistent with the lack of an effect of sterols on the synthesis of

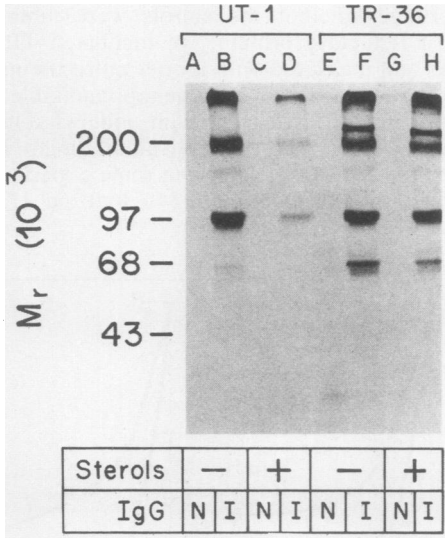

FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ^{35}S-labeled immunoprecipitates from UT-1 cells and TR-36 cells incubated in the absence or presence of sterols as indicated. Cells were set up for experiments by the standard method. UT-1 cells were maintained in the continuous presence of 40 μM compactin. On day 4, each monolayer received 1.5 ml of medium A supplemented with 10% lipoprotein-deficient serum in the absence or presence of 1 μg of 25-hydroxycholesterol per ml plus 10 μg of cholesterol per ml. After incubation for 11 h, each monolayer was washed with 3 ml of phosphate-buffered saline and then received 1.3 ml of methionine-deficient medium (7.5 μM methionine) containing 10% lipoprotein-deficient serum and 154 μCi of [^{35}S]methionine per ml in the absence or presence of 1 μg of 25-hydroxycholesterol per ml plus 10 μg of cholesterol per ml. After the cells were pulse-labeled for 30 min, portions of the cell extracts (10% of a dish of UT-1 cells and 25% of a dish of TR-36 cells) were immunoprecipitated with nonimmune (N) immunoglobulin G (IgG) or immune (I) antireductase IgG as indicated and subjected to sodium dodecyl sulfate-gel electrophoresis. The gels were exposed to X-ray film for 91 h. The position of migration of molecular weight standards is indicated.

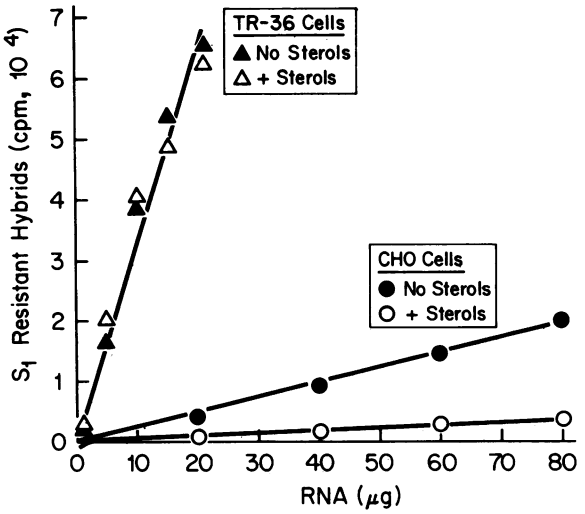

FIG. 6. Measurement of reductase mRNA in CHO cells and TR-36 cells incubated in the absence or presence of sterols. Cells were set up for experiments by the standard procedure except that 100- by 15-mm dishes were used. On day 5, each dish received 8 ml of medium A supplemented with 10% lipoprotein-deficient serum in the absence or presence of 1.5 μg of 25-hydroxycholesterol per ml plus 15 μg of cholesterol per ml. After incubation for 5 h, the cells were harvested for RNA analysis and measurement of reductase activity. Total cellular RNA was hybridized with a single-stranded ^{32}P-labeled reductase cDNA probe, and the hybrids were subjected to S1 nuclease analysis as described in the text. A blank value of 0.3 × 10^4 cpm (representing the amount of S1-resistant hybrids formed in the absence of RNA) was subtracted from each value. The values of reductase activity were as follows: CHO cells incubated without sterols, 0.67 U/mg protein; CHO cells with sterols, 0.13 U/mg protein; TR-36 cells without sterols, 1.4 U/mg protein; and TR-36 cells with sterols, 0.80 U/mg protein.

reductase protein. The mRNA was quantified by solution hybridization to a single-stranded, uniformly ^{32}P-labeled reductase cDNA fragment followed by S1 nuclease digestion and precipitation with trichloroacetic acid. In this highly sensitive assay, cDNA fragments that hybridize to reductase mRNA are protected from digestion with S1 nuclease and can therefore be precipitated by trichloroacetic acid. For each assay we performed a concentration curve of cellular RNA to ensure that the amount of S1-protected fragments was directly proportional to the RNA input (Fig. 6). As a positive control in the same experiment, we measured the level of reductase mRNA in CHO cells incubated in the absence or presence of sterols. When RNA was obtained from CHO cells grown in the absence of sterols, the amount of S1 nuclease resistant hybrids was ~250 cpm/μg of RNA. When the induced CHO cells were incubated with sterols for 5 h, the amount of reductase mRNA was reduced by 80%. TR-36 cells had 15-fold more reductase mRNA than did CHO cells when grown in the absence of sterols. When sterols were present for 5 h before harvest, there was no reduction in the amount of reductase mRNA (Fig. 6).

In contrast to the lack of effect on synthesis of reductase, sterols had a definite effect on the rate of degradation of reductase protein in the TR-36 cells. Figure 7A shows results of an experiment in which TR-36 cells were allowed to synthesize HMG CoA reductase in the presence of [^{35}S]methionine and then switched to unlabeled methionine and incubated in the absence or presence of exogenous sterols. At different intervals, the amounts of [^{35}S]methionine

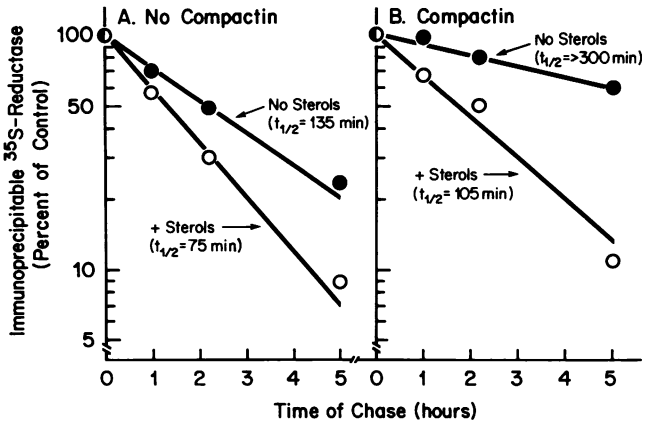

FIG. 7. Effect of sterols on the turnover of ^{35}S-labeled HMG CoA reductase in TR-36 cells cultured in the absence or presence of compactin. Cells were set up for experiments by the standard procedure. On day 3, each monolayer received 2 ml of medium A supplemented with 10% lipoprotein-deficient serum in the absence (A) or presence (B) of 40 μM compactin. Sixteen hours later, each monolayer was washed once with 3 ml of phosphate-buffered saline, after which the cells were pulse-labeled with 100 μCi of [^{35}S]methionine per ml in 1.5 ml of methionine-deficient medium (6 μM methionine) containing 10% lipoprotein-deficient serum and 40 μM compactin as indicated. After a 30-min pulse, each monolayer was washed once with 3 ml of Ham F-12 medium and then chased in 2 ml of medium A containing 300 μM methionine, 10% lipoprotein-deficient serum, and 40 μM compactin as indicated in the absence or presence of 1 μg of 25-hydroxycholesterol per ml plus 10 μg of cholesterol per ml. At the indicated chase time, cells were harvested and solubilized, and portions were processed for precipitation by trichloroacetic acid and immunoprecipitation by nonimmune and antireductase IgG. Each data point represents the average of duplicate dishes. The 100% of control values for total ^{35}S-labeled cell protein in the absence and presence of compactin were 51,500 and 50,400 dpm/μg protein, respectively. The 100% of control values for ^{35}S-labeled reductase (calculated by subtracting the nonimmune IgG immunoprecipitate value from the antireductase IgG immunoprecipitate value) in the absence and presence of compactin were 119 and 123 dpm/μg protein, respectively. The average protein content per dish was 73 μg.

in total cell protein and immunoprecipitable HMG CoA reductase were measured (Fig. 7A). The addition of sterols had no effect on the degradation of total cell protein (data not shown). On the other hand, the sterol mixture produced almost a twofold increase in the rate of degradation of previously synthesized HMG CoA reductase (Fig. 7A). The half-life of the enzyme was 135 min in the absence of sterols and was reduced to 75 min in the presence of the sterol mixture.

In UT-1 cells, which are grown in the continual presence of compactin, the turnover of HMG CoA reductase is retarded (half-life, 13 h) (10), as compared with the turnover in other types of cells grown in the absence of compactin (half-life, 2 to 4 h) (3). To determine whether compactin prolongs the half-life in TR-36 cells, we performed the same type of experiment as in Fig. 7A, but in this case we preincubated the cells in the presence of 40 μM compactin. The data show that in the presence of compactin, the half-life of HMG CoA reductase was greatly prolonged to >300 min (Fig. 7B). When sterols were added, the half-life of reductase was reduced more than threefold to 105 min (Fig. 7B).

Similar results for the half-life of HMG CoA reductase in TR-36 cells grown in the absence or presence of compactin were obtained in a total of seven experiments similar to the one shown in Fig. 7. The results of these experiments are summarized in Table 1.

TABLE 1. Half-life of HMG CoA reductase in TR-36 cells cultured under different conditions[a]

Compound	Parameter			
	Half-life (h)		Stimulation of degradation rate by sterols (fold)	No. of expt
	Mean	Range		
No compactin			1.9	3
− Sterol	2.1	1.8–2.3		
+ Sterol	1.1	1.0–1.3		
40 μM compactin			7	4
− Sterol	12.6	7.8–20		
+ Sterol	1.8	1.5–2.0		

[a] TR-36 cells were set up for experiments, incubated in the absence or presence of 40 μM compactin, pulse-labeled with [^{35}S]methionine for 25 to 45 min, and chased for 3.75 to 10 h with the indicated concentration of compactin in the absence or presence of 1 μg of 25-hydroxycholesterol per ml plus 10 μg of cholesterol per ml, as described in the legend to Fig. 7. The amount of immunoprecipitable ^{35}S-labeled reductase was quantified at various times of chase, and these values were used to calculate a half-life as shown in Fig. 7.

An action of sterols that occurs concomitantly with the suppression of HMG CoA reductase is the stimulation of acyl-CoA:cholesteryl acyltransferase, an enzyme of the endoplasmic reticulum that synthesizes cholesteryl esters (13). Thus, in the current studies, we tested the ability of TR-36 cells to synthesize cholesteryl esters in response to sterols. In TR-36 cells, although sterols and LDL had a diminished ability to suppress reductase activity, these agents nevertheless stimulated the acyl-CoA:cholesteryl acyltransferase enzyme in a normal fashion (Fig. 8).

DISCUSSION

In the current studies, we used the technique of calcium phosphate-mediated DNA transfection to introduce a cloned cDNA for HMG CoA reductase into UT-2 cells, a mutant

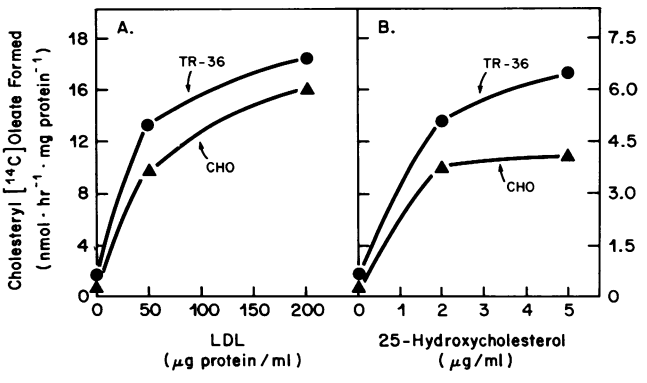

FIG. 8. Stimulation of cholesteryl [^{14}C]oleate formation in TR-36 and CHO cells after incubation with LDL (A) or 25-hydroxycholesterol (B). Cells were set up for experiments by standard procedures. On day 5, each monolayer received 2 ml of medium A containing 5% lipoprotein-deficient serum and the indicated concentration of either LDL (A) or 25-hydroxycholesterol (B). After incubation for 5 h at 37°C, the cells were pulse-labeled for 2 h with 0.1 mM [^{14}C]oleate-albumin (9755 dpm/nmol), after which the cells were harvested for measurement of their content of cholesteryl [^{14}C]oleate. Each value represents the mean of triplicate incubations.

line of CHO cells that lacks functional reductase activity. HMG CoA reductase lends itself well to such studies because efficient methods are available to select for cells that either express or do not express HMG CoA reductase (3, 14). These techniques allowed the earlier isolation of UT-2 cells, which do not express detectable amounts of HMG CoA reductase activity (22). They have now permitted the isolation of TR-36 cells, which have integrated multiple copies of the reductase cDNA into their genomes.

In the initial characterization of TR-36 cells, we made the following observations: (i) TR-36 cells integrated multiple copies of the reductase plasmid; (ii) TR-36 cells produce relatively large amounts of reductase mRNA as compared with CHO cells; (iii) in TR-36 cells, the levels of reductase mRNA and the rate of synthesis of reductase protein are not regulated by 25-hydroxycholesterol or LDL-derived cholesterol; (iv) reductase activity is nevertheless reduced by 50 to 60% when sterols are added in the form of 25-hydroxycholesterol or LDL; and (v) this regulation is explained by a sterol-mediated increase in the rate of degradation of HMG CoA reductase molecules.

Results of previous studies of cultured CHO cells (4), UT-1 cells (10), avian myeloblasts (29), and rat hepatocytes (9) have indicated that LDL, sterols, or mevalonate decrease the amount of HMG CoA reductase by accelerating its degradation, as well as by reducing its rate of synthesis. However, since the acceleration of degradation was always accompanied by a decrease in synthesis, it was difficult to exclude the possibility that the accelerated degradation might be a secondary consequence of a reduced rate of synthesis of the enzyme. Because the transfected reductase cDNA in TR-36 cells is not under the control of the reductase promoter, its transcription is not reduced by sterols. Accordingly, the effect of sterols on enzyme degradation can be dissociated from the effect on mRNA production and protein synthesis.

The effect of sterols on reductase degradation was greatest when the TR-36 cells were grown in the presence of compactin. Under these conditions, the cells cannot synthesize their own sterols or other nonsterol substances derived from mevalonate (3). As a result, the rate of degradation of reductase slows even more than it does when the cells are grown in lipoprotein-deficient serum. When sterols are added to these compactin-treated cells, degradation of reductase is accelerated by as much as sevenfold (Table 1).

In a total of five experiments in which reductase mRNA levels were measured by the quantitative S1 nuclease technique (Fig. 6), we found that the TR-36 cells had an average of 18-fold more reductase mRNA than did CHO cells (range, 11- to 32-fold). In the same experiments, the amount of reductase activity was only twofold higher in the TR-36 cells than in the CHO cells (range, 1.5- to 3.5-fold). In preliminary experiments, we observed that the relative rate of synthesis of reductase from [^{35}S]methionine in TR-36 cells is only about twofold higher than in CHO cells. These data suggest that the reductase mRNA derived from pRed-227 is not translated as efficiently as the natural reductase mRNA. The pRed-227 mRNA has two AUG codons upstream of the AUG codon that is used to initiate translation of the reductase protein (5, 26). In some of the endogenous reductase mRNAs, the region containing these upstream AUG codons is spliced out of the mRNA (26; Reynolds et al., manuscript in preparation). Thus, it is likely that some of the endogenous mRNA molecules in CHO cells may be translated more efficiently than the mRNA produced from pRed-227 in the TR-36 cells.

The current studies focus attention on potential mechanisms by which sterols might stimulate the degradation of HMG CoA reductase protein. One possible mechanism involves the potential interaction of sterols with the complex hydrophobic membranous domain of the reductase. The amino acid sequence of the enzyme, which was deduced from the nucleotide sequence of the cDNA (5), indicates that the NH_2-terminal one-third of the protein is strongly hydrophobic and is predicted to contain seven regions that span the membrane of the endoplasmic reticulum (16a). Results of proteolysis experiments have shown that this portion of the enzyme is tightly embedded in the membrane of the endoplasmic reticulum (16, 16a). The COOH-terminal two-thirds of the enzyme is water soluble and projects into the cytoplasm. This COOH-terminal portion of the reductase can be released from endoplasmic reticulum membranes by proteolysis, whereupon it retains full catalytic activity (16, 16a). What, then, is the function of the membraneous domain? We suggest that one function of this domain is to bind sterols or other mevalonate-derived substances and thereby modulate the rate of degradation of the reductase.

The rate of turnover of HMG CoA reductase in cultured cells and in rat livers in the presence of sterols is much faster than the rate of turnover of other endoplasmic reticulum proteins (3). This suggests that there is some specific mechanism for removing HMG CoA reductase from the endoplasmic reticulum membrane. The binding of sterols to the membranous domain of the reductase might cause the reductase molecules to cluster in a specialized region that buds from the endoplasmic reticulum and is destined for degradation, perhaps in lysosomes. Alternatively, the binding of sterols to the membrane domain might cause a conformational change in the reductase that exposes it to some cytosolic or membrane-bound protease. It should be possible to test these various hypotheses by transfecting UT-2 cells with a cDNA from which the nucleotides encoding the membranous domain of reductase have been deleted.

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